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REMEDIES FOR HYPONUTRITION STATUS

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Abstract of **WO02060472**

Remedies for diseases with hyponutrition status such as inappetence, cachexia or malignant diseases and prostration caused by weight loss in association with infection or inflammatory diseases. These remedies contain as the active ingredient ghrelin or its analogs.

Specification

Hyponutrition symptom disease therapeutic agent.

The Field of the Technology

This invention relates to the following, namely, a novel therapeutic agent for hyponutrition symptomatic diseases. More particularly, this invention relates to a therapeutic agent containing as effective ingredient ghrelin or ghrelin analogue for disease showing hyponutritional symptoms. Moreover, it relates to a novel feeding aberration or metabolic error therapeutic agent using an antagonist or agonist of ghrelin.

Background Technology

As far as body weight control is concerned, the balance between food consumption and energy consumption holds the key, and the balance between the two may cause obesity or emaciation. Since it was made clear that leptin discovered in 1994 was concerned with heart and soul of body weight control as an adiposity (fat mass accumulation) signal, many new peptides participating in appetite control located downstream of leptin have been found. In particular a neuropeptide group derived from hypothalamus regarded only as an individual separate function till then was realised to function downstream of leptin, and furthermore to carry out a concise information exchange between one neuropeptide group and another.

Among these neuropeptides, neuropeptide Y (NPY), orexins, motilin, melanin-concentrating hormone: MCH and agouti-related protein (AGRP) are known to be substances elevating appetite. Moreover, as substances inhibiting appetite, α-melanocyte-stimulating hormone (α-MSH), corticotropin-releasing factor (CRF), cocain-and amphetamine-regulated transcript (CART) and cholecystokinin (CCK) are known. These peptides participate in the physiological mechanism controlling the movement of the gastrointestine, and are thought to influence energy homeostasis.

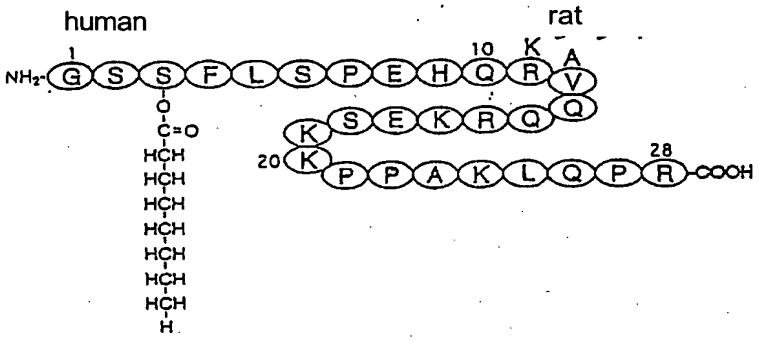
In particular, NPY is a neurotransmitter consisting of 36 amino acids, and it appears abundantly in the hypothalamus which is regarded as a feeding centre. NPY is produced in the hypothalamus arcuate nucleus (ARC), and it is mainly secreted at paraventricular nucleii (PVN) via axons, and influence is exerted on feeding. When NPY is administered centrally, a powerful hyperphagia action is demonstrated

(Schwartz, MW et al, Am. J. CIin. Nutr., 69: 584,1999), however, on peripheral administration, there is no relation to feeding or even conversely, an inhibiting tendency is demonstrated. In the same way this phenomenon is found with other PP family peptides. The various kinds of physiological actions caused by NPY are carried out via NPY receptors. As far as NPY receptors are concerned, five subtypes (Y1, Y2, Y4, Y5, Y6) are cloned at present, and the basic structures thereof are the seven times transmembrane type G protein coupling receptors. Y5 receptors from the results of investigations of ligand bond specificity and food promotion activity, and YI receptor from the results of analysis including antagonist administration experiments, have been reported to be receptors which are closely connected with feeding control (Inui A., Trends Pharmacol Sic 20: 43146, 1999 and the like).

On the other hand, growth hormone (GH) is a hormone secreted from the anterior pituitary gland, and secretion thereof is concisely controlled, whereby stimulation is received by hypothalamus growth hormone releasing hormone (GHRH) and inhibited by somatostatin. Recently, a GH secretion control mechanism using a different pathway from GHRH and somatostatin has become clear. The GH secretion control mechanism of this alternative pathway has been developed by studying the growth hormone secretagogue (GHS) which is a synthetic compound having secretion promotion activity for GH. GHS acts using a different pathway from GHRH. In other words, GHRH activates GHRH receptor and thereby increases intracellular cAMP concentration, while on the other hand, GHS activates a different receptor from GHRH receptors and thereby increases intracellular Ca++ ion concentration via the intracellular IP3 system. The structure of GHS-R which is the receptor where this GHS acts was elucidated using expression cloning method in 1996 (Howard A.D. et al, Science, 273: 974-977,1996). GHS-R is a typical G protein coupling type receptor penetrating seven cell membranes and is mainly present in the hypothalamus and pituitary gland.

Moreover, because receptors which bond GHS, a synthetic compound not present in in vivo, are present, a search was made of an endogenous ligand which was activated by bonding to this GHS-R. As a result, as specific ligand for GHS-R, Ghrelin was refined from stomach of rat and identified (Kojima M. et aL Nature, 402: 656-660, 1999).

Ghrelin is a peptide consisting of amino acid 28 residue and the third serine residue is n-octanoylated. Moreover, human ghrelin is different from rat ghrelin at the amino acid 2 residue. The structural formula of rat and human ghrelin are shown below.



Chemically synthesized ghrelin, in the order of nanomoles, has intracellular Ca^{++} increasing activity for CHO cells expressing GHS-R and activity releasing growth hormone in primary culture pituitary gland cells. Moreover, even in vivo in rats, it increases growth hormone in the blood. The mRNA of ghrelin is markedly apparent in the stomach and also ghrelin is present in blood. Moreover, GHS-R is present in the hypothalamus, heart, lung, pancreas, small intestine and adipose tissue (aforesaid Kojima). Moreover it has been reported that ghrelin has a feeding promoting action (Wren et al, Endocrinology, 141(11): 4325-4328, 2000). From these findings, it is considered that ghrelin is produced in the stomach and carried to the pituitary gland through the blood, and thereafter exerts various actions on the brain and periphery. However, the physiological role thereof has not yet been thoroughly elucidated.

The object of this invention is to elucidate the action of ghrelin in appetite control and the mechanism thereof, and moreover to develop a novel hyponutrition symptom disease therapeutic agent using this. And moreover it is a further object to develop a novel therapeutic agent for feeding abnormalities and metabolic aberration using an antagonist or agonist of ghrelin.

Disclosure of the invention.

In order to attain the said objectives, these inventors carried out assiduous investigations and as a result, discovered that ghrelin demonstrated a marked appetite promotion action through NPY and YI receptors. This invention was completed on the basis of this discovery.

In other words, this invention puts forward a therapeutic agent for diseases showing hyponutrition symptoms containing ghrelin as effective ingredient.
Moreover, this invention puts forward a screening process for agonists or antagonists of ghrelin including the measurement of food consumption, NPY mRNA expression quantity, the bonded quantity of NPY and YI receptors of NPY, oxygen consumption, rate of gastric contents expulsion and activity of vagal nerve by administering a candidate substance to an animal in the presence or absence of ghrelin.

Moreover, this invention puts forward a therapeutic agent anorexia or weight loss disease containing as agonist of ghrelin obtained using the aforesaid process as an effective ingredient.

Moreover, this invention puts forward an obesity preventative agent or therapeutic agent containing an antagonist of ghrelin obtained using the aforesaid process as an effective ingredient.

Brief Description of the Figures

In Figure 1, A shows the amino acid sequence of human ghrelin and human motilin. B shows the amino acid sequence of human ghrelin receptor and human motilin receptor. The same amino acids are indicated by an asterisk.

Figure 2 shows the effect of ghrelin ICV administration on feeding.

Figure 3 shows the effect of mouse ICV administration of ghrelin compared to NPY, AGRP, orexin A, orexin B and MCH.

Figure 4 shows the NPY genetic expression in hypothalamus after ICV administration of ghrelin. The panel at the top shows the Northern blot of hypothalamus NPY mRNA after ghrelin ICV administration. The graph at the bottom shows the Northern blot data using the percentage of control group normalised to G3PDH mRNA.

Figure 5 shows the effect exerted on feeding induced by ghrelin as a result of carrying out pre-treatment with YI receptor antagonist (BIBO3304) and Y5 receptor antagonist (L152804) of NPY.

Figure 6 shows the effect of ghrelin ICV administration exerted on oxygen consumption.

Figure 7 shows the effect of ghrelin IP administration exerted on feeding.

Figure 8 shows the effect of ghrelin IP administration exerted on hypothalamus NPY mRNA expression.

Figure 9 shows the effect of ghrelin IP administration exerted on the rate of gastric contents expulsion.

Figure 10 shows the effect cutting the vagal nerve exerts on the feeding promoting effect of ghrelin.

Figure 11 shows the effect cutting the vagal nerve exerts on afferent activity of stomach vagal nerve on ghrelin administration.

Figure 12 shows the results obtained by examination by Northern blot analysis of expression of ghrelin mRNA in stomach in a lean mouse fasted for 48 hours.

Figure 13 shows the results obtained by examination by Northern blot of expression of ghrelin mRNA in stomach on IP-administration of IL-1 beta and leptin.

Figure 14 shows the results obtained by examination by Northern blot of expression of ghrelin mRNA in stomach of ob/ob obese mouse.

Figure 15 shows the results obtained by examination by Northern blot of expression of ghrelin mRNA in stomach on the repeated administration of leptin to ob/ob mice.

Figure 16 shows the effect exerted on food consumption and body weight on co-administration of ghrelin and IL-1β to fasted lean mouse.

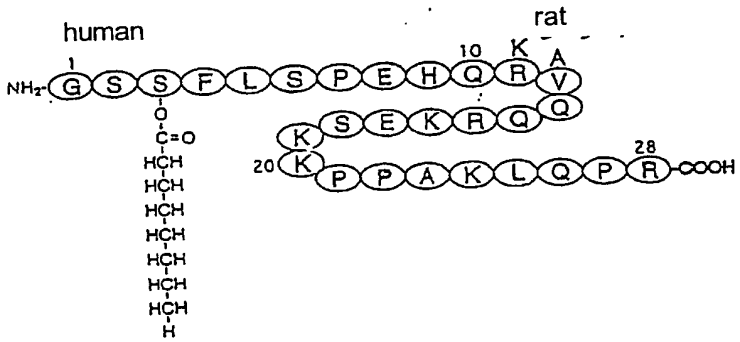
Figure 17 shows the effect exerted on decrease of food consumption and body weight induced by IL-16 on repeated administration of ghrelin.

Figure 18 shows the effect ghrelin exerts on body weight gain in LC-6 transplanted HHM/cahexia model mouse.

Figure 19 shows the effect ghrelin exerts on fat increase in LC-6 transplantated HHM/cahexia model mouse.

Ideal form for Carrying Out the Invention.

Ghrelin used as effective ingredient in this invention is a rat ghrelin or human ghrelin or ghrelin analogue represented by formula 1.



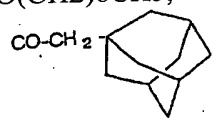
The ghrelin analogues include ghrelins wherein one or more amino acids of the 28 amino acids is missing, substituted or added to as long as the appetite promotion action is still present, and furthermore, includes various derivatives thereof, for example derivatives wherein the peptide constituent amino acids are substituted (including compounds wherein a group, for example alkylene is inserted between amino acids) and also ester derivatives.

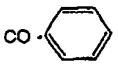
The ghrelin or ghrelin analogue may be a ghrelin produced by any process, for example by separation from cells of a man or rat, and purified, synthetic product and semisynthestic products thereof, and also ghrelins obtained using genetic engineering techniques or the like, and there are no restrictions to these in particular.

An example of such a ghrelin in which one or more amino acids of 28 amino acids is missing, substituted or added thereto, comprises des-Gln14-ghrelin wherein the 14th ghrelin G1n residue is is missing. Rat des-Gin14-ghrelin is produced due to a difference in ghrelin genetic splicing and is present in around a quarter of the ghrelins in rat stomach, and has the same strength of growth hormone release activity as ghrelin.

Moreover, in J. Med. Chem. 2000, 43, 4370-4376, the smallest sequence of ghrelin necessary for activation of human GHSR1a is described, and the the ghrelins described below herein are included in the ghrelin analogue of this invention. Examples include the ghrelins comprising peptides including the amino acids from the N terminal to the the 3rd amino acid and 4th amino acid from the N terminal (preferably the amino acid 4 from the N terminal) among the ghrelin 28 amino acids wherein the side chain of the third amino acid from the N terminal (Ser) is substituted, and derivatives thereof and having an appetite promotion action is nominated.

Examples of the side chain of the third amino acid from the N terminal, other than n-octanoyl which is side chain of ghrelin include acyl groups and substituted alkyl groups (preferably of carbon number 6-18). Embodiments of these side chains include the following:
-CO-(CH2)6CH3, -CO(CH2)9CH3, -CO(CH2)14CH3, -CO-CH=CH-CH=CH-CH=CH-CH3, -CO-CH (CH2CH2CH3)2, -CO-(CH2)6CH2Br, -CO-(CH2)2CONH(CH2)2CH2, -CH2-NH-CO(CH2)8CH3, -CH2-O-CO(CH2)8CH3, -CH2-NH-CO(CH2)6CH3,





Embodying examples of the ghrelin analogues having from the N terminal to the 3-4th amino acid wherein the side chain of the third amino acid from N terminal (Ser) is substituted include the compounds reported in the 37th Peptide Panel Discussion (from 18 to 20 on October in 2000): NH2-(CH2)4-CO-Ser (octyl) -Phe-Leu-NH-(CH2)2-NH2.

When the action mechanism of ghrelin described hereinafter and as clarified by this invention is considered, these ghrelin analogs can also be anticipated to have appetite promotion activity.

In the therapeutic agents for disease showing hyponutrition symptom of this invention, ghrelin or ghrelin analogs may be used in combinations of two or more thereof.

The therapeutic agents for diseases showing hyponutrition symptom of this invention can be administered by a central administration (for example intracerebroventricular administration or spinal

cord cavity injection) or by peripheral administration. Preferably they are applied via a peripheral administration. As described above, NPY and other PP family peptide show no correlation to feeding when administered to the periphery, however the therapeutic agents of this invention show a marked hyperphagia effect even on peripheral administration. Accordingly, the therapeutic agents of this invention can be simply taken with less pain to the patient on administration, and are of far greater merit compared to the prior art appetite regulating peptides.

The ghrelins and ghrelin analogue can be made into conventional oral administration formulations and formulations for parenteral administration on their own or in combination with pharmacologically acceptable carriers or additives using well known formulation techniques. For example, they can be formulated pharmaceutically into solution formulations (injection agents for intra-arterial injections, intravenal injections or subcutaneous injections, nasal drops, syrups and the like), tablets, troche agents, encapsulated formulations, powders, granules, ointments, suppositories and the like. Moreover, they can be used in drug delivery systems (sustained release agents and the like).

The dose of the therapeutic agents of this invention for diseases showing hyponutrition symptoms differ depending on the patient age, body weight and symptoms, and also the administration pathway, and is determined upon diagnosis by a medical doctor. Usually for ghrelins for intravenously administration, the dose is about 0.1 μg-1000 mg, preferably about 0.01mg-100mg, more preferably 0.1mg-10mg per 1 kg weight. However, the dose is not restricted to this range.

The therapeutic agents of this invention can be used for the therapy of disease showing hyponutrition symptoms and in particular are effective in diseases selected from prostration caused by weight loss in association with infection or inflammatory diseases, malignant diseases, cachexia and inappetence. More particularly they are useful as therapeutic agents for weight loss disease of anorexia or accompanied by cachexia. Cachexia is a poor whole body condition with as main symptoms, a gradual progressing weight loss, anemia, skin desiccation or edema, loss of appetite and the like, and it is seen as the terminal stage in very many diseases such as infections, parasitic diseases, malignant tumors and the like. In this specification, terms such as hyperphagia, feeding increase, feeding promotion and the like are interchangeably used as words having equal meaning (?).

Moreover, this invention puts forward a screening process for agonists or antagonists of ghrelin or ghrelin analogues including the measurement of food consumption, NPY mRNA expression quantity, the quantity bonded for NPY and YI receptors for NPY, oxygen consumption, rate of gastric content expulsion or activity of vagal nerve by administering candidate substances to an animal in the presence or absence of ghrelin. As embodiments of the methods for measurement, it is for example possible to use a process in accordance with this specification, but there is no restriction however to this.

The agonists of the ghrelin or ghrelin analogue obtained by the aforesaid screening process can be used as effective ingredients in the anorexia or weight loss disease therapeutic agents of this invention.

Moreover, the antagonists of ghrelin or ghrelin analogue obtained by the aforesaid screening process can be used as the effective ingredient of obesity preventative agents or therapeutic agents of this invention. As will be shown in the following Examples, feeding promotion induced by ghrelin was hindered significantly by carrying out pre-treatment with a YI receptor antagonist of NPY. Accordingly, by administration of an antagonist of ghrelin or ghrelin analogue, it is possible not only to treat obesity but also to prevent it.

In accordance with this invention, motilin or a motilin analogue or an agonist thereof can be used as the agonist of the ghrelin or ghrelin analogue, and moreover it is possible to use an antagonist of motilin or an motilin analogue as the antagonist of the ghrelin or ghrelin analogue.

Motilin is a peptide of 22 amino acid residues secreted from endocrine cells in the duodenum and upper jejunum (ltoh, Z., Peptides, 18: 593-608,1997), and it participates in the fasting phase (interdigestive) movement of the gastrointestinal tract, gall bladder contraction and enzyme secretion from the stomach and pancreas. It has been reported that motilin promotes GH secretion, and that movement of the stomach is promoted using a nonpeptidic motilin agonist of (above mentioned, ltoh). As shown by A in Figure 1, human ghrelin and human motilin demonstrate a 36 % amino acid shared identicality (access number A59316 and P12872). Furthermore as shown by B in Figure 1, human ghrelin receptors demonstrates a 50 % amino acid identicality as a whole with human motilin receptors (access number Q92847, Q92848 and Q43193). Moreover Tomasetto et al. recently isolated a novel peptide from mouse stomach, but this is identical with ghrelin and has been called a motilin related peptide (Tomasetto

C. et al., Gastroenterology, 119: 396-405, 2000). In the light of the sequence homology between ghrelin and motilin and the sequence homology between ghrelin receptors and motilin receptors, motilin or motilin analogues or agonists thereof can be used as agonists of ghrelin and ghrelin analogues, and moreover it is possible to use antagonists of motilin and motilin analogue as antagonist of ghrelin and ghrelin analogues.

The mechanism controlling the balance between feeding and energy is complicated, and it has not yet been thoroughly elucidated. So far, it has been shown that many peptides including NPY, AGRP, orexins, MCH, beacon, melanocyte stimulating hormones (MSH), neuromedine U, cocaine- and amphetamine-regulation transcripts (CART), corticotropin-releasing factors (CRF) and leptin, influence energy homeostasis. As described above, NPY, a peptide comprising 36 amino acids, is one of the components comprising a key in the feeding stimulation system and body weight control. In studies to day, centrally administered NPY stimulates feeding in rodents and lowers the metabolic rate (Bray G.A. et al, Recent Prog Horm Res, 53: 96-118,1998). According to pharmacological attribute determinations of the receptors using NPY analogues and specific antagonists, any YI receptor and Y5 receptor may be thought to be a NPY feeding receptor.

In accordance with this invention, ICV-administered ghrelin markedly stimulates feeding in the same way as NPY and decreases oxygen consumption, and these effects were blocked with YI receptor antagonist in each case. On immunohistological analysis, the presence of a small amount of ghrelin in the brain has been suggested by amplification using reverse transcription polymerase chain reaction (RT-PCR). In accordance with a prior art report, GHS-R is localised in the arcuate nucleus (ARC) and NPY is synthesised in the arcuate nucleus (Tannenbaus GS. et al., Endocrinology, 139: 4420-4423, 1998). By an in situ hybridisation test, it was demonstrated that GHS-R and NPY are co-localised together in the arcuate nucleus neurons (Guan XM. et al., Brain Res MolBrain Res, 48: 23-29, 1997 and the like). Furthermore it is known that nonpeptidic growth hormone release promoting factor functions in the hypothalamus, changes the electrical activity of the arcuate nucleus neurons, and activates expression of thetranscription factor c-fos (Dickson SI et al., Neuroendocrinology, 61: 36-43, 1995 and the like).

In accordance with this invention, mRNA expression of NPY is increased significantly by the central administration of ghrelin. Accordingly, the ghrelin action mechanism producing the positive energy

balance is thought to be to related to the NPY and YI receptor system in hypothalamus. So far, it has been demonstrated that some peptides increase feeding when administered to the brain (Elmquist JK et al., Nat Neorosci, 1: 445-450, 1998 and the like). However, there have been no reports so far of peptides showing a hyperphagia action on peripheral administration. In accordance with this invention, it is made clear that peripherally administered ghrelin stimulates feeding via NPY and YI receptors. It is has been suggested that a rapid stomach contents discharge is closely related to overfeeding and obesity, while in the same way, a delay in stomach contents discharge is related to loss of appetite and cachexia (Inui A., Cancer Res 59: 4493-4501, 1999 and the like). In this invention, ghrelin increased the rate of gastric contents expulsion significantly in the same way as motilin. In studies to date, it has been reported that cholecystokinin (CCK) has a powerful feeding inhibition effect and a hindering effect on stomach voiding via centripetal activation of the stomach vagal nerve (Schwartz GJ et al., Am J Physiol, 272: R1726-1733, 1997).

In accordance with this invention, it is made clear that the feeding promoting effect of ghrelin is also via the vagal nerve and centripetal activity. As is made clear from an electrophysiological study, the effective dose of ghrelin on intravenous administration to rat is lower than the effective dose of CCK. It has been reported that various kinds of anti-orexin molecules (anorexigenic molecules) including bombesin, IL-1β, leptin and gastrin-releasing peptide (GRP) increase the stomach vagal nerve centripetal discharge rate (as per above, Schwartz and the like). Accordingly, the effect of ghrelin exerted on the vagal nerve activity and feeding is opposite to feeding inhibition molecules, giving support to the concept that the feeding promotion activity is caused to act through the vagal nerve.

Moreover, this invention demonstrates that expression of ghrelin mRNA in the stomach is increased due to a starved state. These results suggest that the increase in ghrelin mRNA in a starved state is, at least partially, a cause of activation of the hypothalamus NPY, and that this as a result causes feeding. If this were to be the case, the stomach is not only a source of production of leptin which forms a satiety signal from the periphery to hypothalamus, but also a source of production of ghrelin which is a feeding stimulation signal. On loss of appetite and cachexia, cytokines such as IL-1 and IL-6 and tumor necrosis factor exert an important influence on the energy balance (Inui A., Cancer Res 59: 4493-4501, 1999 and the like). Moreover in cachexia, a poor whole body condition comprising weight loss as a predominant symptom is caused. Some hormones, including leptin, CRF, CCK and insulin, which are

known to inhibit feeding, are caused to be derived because of cytokines (?). In accordance with this invention, it has been demonstrated that in the stomach, ghrelin mRNA expression is decreased by both IL-1β and leptin and increased in ob/ob mice (mouse made obese by excess eating because of a lack of leptin). When leptin is administered repeatedly, not only the intake of energy but also the expression of ghrelin mRNA are decreased. Accordingly, ghrelin gene expression in the stomach is closely related to the regulation of appetite, and it plays a role in both the development of obesity and a suitable response to a starved state. Moreover, peripherally administered ghrelin reverses weight loss and loss of appetite induced by IL-1β, and improves cachexia conditions. It is known that ghrelin strongly stimulates growth hormone release from the pituitary gland (as per above, Kojima et al.,). When these findings are considered together with the findings of this invention, it is possible that by merely carrying out IP administration of ghrelin, body weight gain can be stimulated and this peptide will contribute to the regulation of adipose tissue and body growth. These inventors are not restricted by specific theory, but ghrelin, rather than the short term diet related orexigen (a counter part of CCK and other diet related satiety factors), may be a factor controlling body weight in the long term, namely a counter part to leptin. So far, growth hormone has been used as a powerful anabolic agent in the treatment of muscle loss related to surgical operation stress, septicemia, glucocorticoid administration, HIV infection and cancer. Growth hormone, at least under specific conditions, stimulates whole body and muscle proteosynthesis. Accordingly, ghrelin would be effective in the therapy of the elderly with decreased growth hormone secretion, and decreasing muscle mass which in many cases is accompanied by loss of appetite.

The therapeutic agents of this invention for diseases showing hyponutrition symptoms containing ghrelin or ghrelin analogue as an active ingredient, induce a positive energy balanced state and body weight gain by promoting feeding, decreasing energy consumption and stimulating growth hormone secretion.

This invention will be described in greater detail by reference to the following Examples. However, this invention is not restricted by these Examples. Various changes and modifications can be made by a person skilled in the art based on the description of this invention, and such changes and modifications are included within the scope of this invention.

Test Materials and Methods.

(1) Animal tests

7 week old ddy male mice (32-35 g) were purchased from JAPAN SLC (Shizuoka, Japan). 10-11 week old obese type (ob/ob) C57BL/6J mice (38-42 g) were purchased from Shionogi Co, Ltd, (Shiga, Japan). These were bred in individually controlled environments (temperature 22±2°C, humidity 55±10 %, light-dark cycle every 12 hours, light cycle starting at 7:00 a.m.). Food and the water were arbitrarily given until otherwise specially stated. The mice were used only one time in each experiment. Rat ghrelin, rat NPY, human agouti-related protein 86-132 (AGRP), mouse orexin A, mouse orexin B and mouse melanin concentrating hormone (MCH) were purchased from the Peptide Institute (0saka, Japan). Recombinant mouse leptin and recombinant mouse IL-1β were purchased from R&D Systems (Minneapolis, USA) and Upstate Biotechnology (New York, USA) respectively. The BIBO3304 was supplied from Boehringer-Ingelheim Pharma (Germany) and also the L152804 and J115814 were from Banyu (Banyu Pharmaceutical Co.,Ltd, Tokyo, Japan). Moreover BIBO3304 and J115814 are YI receptor antagonists for NPY, and L152804 is a Y5 receptor antagonist. Each agent was given by intra-third corebroventricular administration (ICV) by diluting with synthetic cerebrospinal fluid (ACSF) 4 µl immediately before administration or intraperitoneally administered (IP) by dilution with physiological saline 100 µl. Only ACSF or physiological saline was given to control group. Each antagonist was administered simultaneously with ghrelin. The results are shown as the mean value ± SE. Dispersion analysis (ANOVA) was carried out, and the difference between the groups was determined by the t test of Bonferroni. It was assumed that when $P<0.05$, that there was a statistical significant difference.

(2) ICV administration.

In order to carry out the ICV administration, the mice were anaesthetised with pentobarbital sodium (80-85 mg/kg IP) and placed in a stereotaxic apparatus (SR-6, Narishige, Tokyo, Japan) for seven days before the experiment. Holes were opened at a position of 0.9 mm of the side of middle suture at 0.9 mm in front of the apex on each cranial bone using a needle. Cannula (24 gauge) (Safelet-Cas, Nipro, 0saka, Japan) wherein one end was slanted for a length of 3 mm were buried in the third ventricle, and these were prepared for ICV administration. The cannula were fixed to the cranial bone with dental cement and capped with silicone. The insert for the infusion (27 gauge) was attached to a microsyringe by PE-20 tubing. This was introduced into aforesaid fixed cannula without restricting or greatly controlling the behavior of the mouse. On completion of the experiment, dye (Evans blue 0.5 % and

gelatin 5 %) was injected in order to confirm the position of the end of the cannula, and histological testing of the frozen brain section was carried out.

(3) Vagal nerve cutting (truncal vagotomy).

A vagotomy was carried out 4 days before the experiment as follows. The mice were anaesthetised with pentobarbital sodium (80-85mg/kg IP). The central line of the abdominal wall was cut open, and the stomach was covered with sterilized gauze moistened with warm aqueous sodium chloride. The lower part of the oesophagus was exposed, and the front branch and posterior branch of the vagal nerve were cut. As the final part of the procedure, the abdominal wall was double sewn. In the same way, for a sham (false) operation mouse, the vagal nerve stem was exposed to light but the cutting was not carried out. The mice with the cut vagal nerve and the mice with the sham operation were bred using a full nutrition fluid diet (Oriental Yeast Co. Ltd. Tokyo, Japan).

(4) Feeding test.

The examination was started at 10:00. Before the feeding test was started, food and water were freely fed to the mice. However, on examination to examine the effect of IP co-administration of ghrelin and IL-1β on feeding, food was not given to the mice for 16 hours, and only water was freely fed. The measurement of food consumption was determined by subtracting the quantity of food remaining 20 minutes, 1 hour, 2 hours and 4 hours after the ICV or IP administration, from the amount of food given and measured beforehand. In the lean mouse with unrestricted feeding, IP-administration was repeated for five days with ghrelin (3 nanomole / mouse), IL-1β (5 picomole / mouse) or physiological saline. The injections were carried out to the mice every day at 7:00 and 19:00. The food consumption and body weight were measured every day, and the condition of their coat was observed.

(5) Isolation of RNA and Northern blot analysis.

RNA was isolated using an Rneasy Mini Kit (Qlagen, Tokyo, Japan) from the hypothalamus block and stomach. All the RNA was modified with formaldehyde, subjected to electrophoresis with 1 % agarose gel, and it was plotted on a Hybond N+ membrane (Amersham Pharmacia Biotech AB, Uppsala,Sweden) (Ueno N. et al., Gastroenterology, 117: 1427-1432, 1999). The membrane was hybridised with 32P-labeled cDNA probe, and the NPY mRNA in the hypothalamus was measured and the ghrelin mRNA in stomach measured by hybridising with digoxigenin labelled cDNA probe. All the hybridised signal was

measured by densitometry (Image Master ID Elitever 3.0, Amersham Pharmacia Biootech AB, Uppsala, Sweden). The data was normalised to glyceraldehyde triphosphoric acid dehydrogenase (G3PDH) mRNA, and expressed as a percentage of the control group.

(6) NPY gene expression.

The mice were fasted for 24 hours. Ghrelin (1 nanomole / mouse) or ACSF was administered to mice which had been set up for ICV-administration beforehand at intervals of 12 hours during the fasting period, and the mice were killed by cervix dislocation 30 minutes after the third last administration. Ghrelin (3 nanomole / mouse) or physiological saline was administered to mice which had been set up for IP-administration beforehand at intervals of eight hours, and the mice were killed by cervix dislocation 30 minutes after the 4th and last administration. The brain block was promptly removed surgically, and frozen with dry ice, and thereafter stored at -80°C until preparation of the Northern blot.

(7) Ghrelin gene expression.

Lean mice were fasted for 48 hours. IL-1β (5 picomole / mouse), leptin (3 nanomole / mouse) or physiological saline were IP-administered to the the fasted mice every 12 hours during the fasting period, and the mice were killed by cervix dislocation at 30minutes after 5th and last administration. In ob/ob mice which had not been fasted, leptin (3 nanomole / mouse) or physiological saline was administered repeatedly for seven days. The injections were carried out to mouse every day at 7:00 and 19:00, and the mice were killed by cervix dislocation at 30 minutes after the last administration. The stomachs were promptly surgically removed, frozen with dry ice and stored at -80°C until preparation of the Nothern blot.

(8) Oxygen consumption.

The oxygen consumption was measured using an O_2/CO_2 metabolism measurement system (Model MK-5000, Muromachikikai, Tokyo, Japan) at 22°C (as per above, Ueno N. et al.). The chamber capacity was 560 ml and the flow rate of air to the chamber was 500 ml / minute. Samples were withdrawn every three minutes and a standard gas sample was withdrawn every 30 minutes. The mice were introduced into the chamber in the light cycle, and were not given food or water, and the ghrelin was ICV-administered (0.3-1 nanomole / mouse at 10.00) in the presence or absence of BIBO3304 (5 nanomole / mouse), and the oxygen consumption was measured two hours after this.

(9) Rate of gastric contents expulsion

Before the measurement and examination of the rate of the gastric contents expulsion, the mice were fasted for 16 hours but fed water freely. Food pellets in an amount measured beforehand were fed freely to the fasted mice for one hour and thereafter the ghrelin was administered. The mice were fasted again for 1 or 2 hours after the administration. The weight of the feed was determined by measuring the remaining pellets. The mice were killed by cervix dislocation 2 or 3 hours after starting the test. The stomach was promptly exposed by a celiotomy and the pylorus and cardia were quickly ligatured and following elimination, a measurement of the dried contents weight was measured. The contents were dried using a vacuum lyophilization system (Model 77400, Lebconco, Kansas, USA). The rate of gastric contents expulsion was calculated by following equation.

Rate of gastric contents expulsion (%) = {1-(dry weight of food recovered from stomach / feeding weight)} x 100.

(10) Electrophysiological study

Male Wistar rats (300 g) were anaesthetised with urethane (1g/kg IP), and a trachea cannula was introduced. Using a dissection microscope, the neurofilaments were excised from the peripheral cleavage end of the stomach branch (gastric branch) of the vagal nerve parts, and afferent nerve activity was recorded with a pair of silver wire electrodes. A rate meter (five second interval) was used in order to observe the change of nerve activity with time (Niijima A, J. Nutr, 130: 9719-973S, 2000). Ghrelin (3-300 femtomole / rat) administration was performed through a small catheter introduced into the inferior vena cava (IV). The effect of the ghrelin gave to vagal nerve activity was measured by comparison of the mean value of impulse every five seconds for 50 seconds before and after the injection. The results was expressed as the mean value ± SE. ANOVA and a Scheffe assay was carried out, and the difference between the groups was evaluated. If $P<0.05$, an assessment of statistical significance was made.

Example 1

The effect ghrelin ICV administration exerts on feeding.

In this example, the effect of a mouse cerebral ventricle (ICV) administration of ghrelin was investigated. ACSF (control) or ghrelin (0.003-1 nanomole / mouse) was ICV-administered to lean mice which had not been fasted. The food consumption was investigated at 20 minutes, 1 hours, 2 hours and 4 hours after the agent administration. The obtained results are shown in Figure 2. The results are expressed as the mean ± SE, wherein n denotes the animal number. *P<0.05 and **P<0.01 are significant differences compared with the control group in accordance with the t test of Bonferroni. The ghrelin markedly and significantly increased food consumption in a dose dependent manner. At 24 hours after ICV administration, the cumulative food consumption was also increased in mouse to which had been administered 1 nanomole ghrelin, but this was not statistically significant difference (6.31 ± 0.10 g : 5.68 ± 0.21 g (control): p<0.076).

Example 2

Effect of ghrelin ICV administration compared to other peptides

The effect of mouse ICV administration of ghrelin compared to NPY, AGRP, orexin A, orexin B and MCH was investigated using 1 nanomole / mouse in the same way as in Example 1. The results are shown in Figure 3. The feeding increase ability at four hours was in the order of NPY> ghrelin >AGRP> orexin A> orexin B>MCH. Accordingly the ghrelin was more powerful than each of the appetite promoting peptides apart from NPY.

Example 3

Effect of ghrelin ICV administration exerted on NPY gene expression

In order to investigate the probability of ghrelin acting via the NPY pathway, the expression of NPY genes in the hypothalamus after an ICV administration of ghrelin was examined using the aforesaid process. The obtained results are shown in Figure 4. The top panel shows the northern blot of the hypothalamus NPY mRNA after ghrelin ICV administration. The lower graph represents this as a percentage of control group with the data of Northern blotting normalised to glyceraldehyde triphosphoric acid dehydrogenase (G3PDH) mRNA. The ghrelin caused an increase in the expression of NPY mRNA of 58 %.

Example 4

The effect pretreatment by an NPY receptor antagonist exerts on ghrelin induction feeding increase

An investigation was made as to whether pre-treatment with the YI receptor antagonists (BIBO3304 and J115814) and Y5 receptor antagonist (L152804) of NPY imparted an effect on the feeding induced by ghrelin or not. BIBO3304 and J115814 which were the YI receptor antagonists (5 nanomole / mouse = ICV administration) hindered feeding induced by ghrelin (1 nanomole / mouse = ICV administration) significantly. On the other hand, the L152804 Y5 receptor antagonist (5 nanomole / mouse = ICV administration) did not demonstrate this effect (Figure 5). Accordingly it was thought that ghrelin acts via the YI receptor of NPY.

Example 5

The effect ICV administration of ghrelin exerts on oxygen consumption

The effect ICV administration of ghrelin exerts on oxygen consumption was investigated using the aforesaid process. The obtained results are shown in Figure 6. The ghrelin decreased oxygen consumption by the same dose promoted feeding (0.3-1 nanomole / mouse = ICV administration), but this was obstructed by pre-treatment with YI receptor antagonist (BIBO3304: 5 nanomole / mouse = ICV administration). Moreover, ghrelin demonstrated a tendency for increasing the respiratory quotient (RQ) in mice administered with 1 nanomole ghrelin at one hour (7.24 ± 6.96 %) and two hours (5.18 ± 6.01 %) after ICV administration, but not to a statistically significant different extent.

Example 6

Effect ghrelin IP administration exerts on feeding and NPY mRNA expression

An investigation was carried out as to whether IP-administered ghrelin showed a similar feeding promoting effect in lean mice which had not been fasted. The IP administration of ghrelin markedly increased feeding at 3 nanomole / mouse (Figure 7). The accumulative food consumption was significantly higher after 24 hours of IP-administration of ghrelin of 3 nanomole / mouse (6.68±0.16 g : 6.10±0.17 g (control): $P<0.05$). This feeding promoting activity was blocked by ICV administration of BIBO3304 which is a YI receptor antagonist, but it was not blocked by the L152804 Y5 receptor antagonist. The mRNA expression of hypothalamus NPY after IP administration of ghrelin was investigated using northern blotting, whereupon an expression increase of 12 % was found (Figure 8: the

saline in Figure 8 shows the results for a group administered with aqueous sodium chloride as a control group).

Example 7

Effect ghrelin IP administration exerts on the rate of gastric contents expulsion

Using the aforesaid process, an investigation was made as to whether ghrelin increases the rate of gastric contents expulsion. Figure 9 shows the rate of gastric contents expulsion at one hour and 2 hours after ICV-administering ghrelin (0.3-1 nanomole / mouse). With ICV or IP administration, the ghrelin had significantly increased the rate of gastric contents expulsion at 1 hour after administration (30.16±3.70 % (3 nanomole) : 20.34 ±2.27 % (control): $p<0.05$). Accordingly ghrelin elevates the movement of the stomach in the same way as in motilin.

Example 8

Effect vagal nerve cutting exerts on the feeding promoting effect of ghrelin, NPY mRNA expression and afferent nerve activity of stomach vagal nerve

Using the aforesaid process, an investigation was carried out as to whether in mice to which vagal nerve cutting had been applied, the feeding promoting effect of ghrelin was connected to a pathway through the vagal nerve. Vagal nerve cutting is an invasive operation. However, the feeding promoting effect induced by the IP administration of ghrelin was caused to disappear by this operation (Figure 10: wherein in Figure 10, Sham denotes the sham operation group, Vagotomy denotes the group subject to vagal nerve cutting). The significant increase in hypothalamus NPY mRNA expression induced by IP-administered ghrelin was also caused to disappear by this operation (the data is not shown). In an electrophysiological study carried out using the aforesaid process, an IV administration of ghrelin significantly decreased the afferent nerve activity of stomach vagal nerve (Figure 11).

Example 9

Expression of ghrelin mRNA in stomach

Expression of ghrelin mRNA in stomach was investigated using Northern blot analysis. As shown in Figure 12, ghrelin mRNA was significantly increased with 48 hours fasting compared to the mice which had not been fasted in the control (16 %) (wherein, in Figure 12, Fed denotes the mouse group which had not been fasted and Fast denotes the mouse group which had been fasted).

IL-1beta and leptin were IP-administered in order to investigate whether any catabolite was giving influence to ghrelin mRNA expression in stomach. In each case, as shown in Figure 13, IL-1 beta and the leptin decreased expression of ghrelin mRNA in stomach significantly (23±2.8 % for IL-1 beta and 22±3.5 % for leptin).

Moreover, Northern blot analysis of the stomach of ob/ob obese mice was carried out. Compared with lean mice which had had food in a arbitrary amount, the ghrelin mRNA expression in the ob/ob mice was increased significantly (19 %) (Figure 14). Leptin significantly decreased the expression of ghrelin mRNA in both ob/ob mice and lean mice (17 ± 3.2%: $P<0.01$). When leptin was administered to ob/ob mice repeatedly, ghrelin mRNA was decreased significantly (31 %) compared with a control group which had been given aqueous sodium chloride, and moreover the food consumption and body weight had decrease simultaneously (Figure 15). No detection of expression of ghrelin mRNA was detected in the Northern blot of the hypothalamus in either the ob/ob or the control mice.

Example 10

Effect of co-administration of ghrelin and IL-1 beta on food consumption and body weight.

The effect exerted on the food consumption and body weight on administration with ghrelin and IL-1 beta was examined. As shown in figure 16, the feeding decrease induced by IL-1β was blocked by ghrelin. Moreover, when ghrelin was administered repeatedly, the food consumption and decrease in body weight induced by IL-1β was reversed (figure 17).

Moreover, the hair coat was dishevelled in the group administered only IL-1 beta, and as deterioration in the whole body condition was observed, but in the group administered with both ghrelin and IL-1 beta, the hair coat was improved, and could be seen that the ghrelin had improved the poor whole body condition accompanying cachexia.

Example 11

Influence of ghrelin with respect to LC-6 transplantation HHM/cahexia model mouse.

As model animals, HHM model mice with transplanted human lung cancer cells strain LC-6 were used. It is known that this model mouse demonstrates a weight loss accompanied by cachexia (WO98/13388).

Ghrelin was administered to the mice comprising 6 animals per group at 3, 0.3, 0 nmol/ individual 10 times (twice a day, every 12 hours for five days). Separately, a normal group (n=5) without the transplanted tumour was prepared.

The body weight was measured at 0-5th day, and moreover the fat weight around the testes was measured at day 5.

The obtained results are shown in Figure 18 and figure 19. An increase in body weight was observed for three days after starting administration in the 3 nmol treated group compared to the 0 nmol treated group, and this lasted until the 5th day. Moreover, the increase in fat weight was observed in the 3 nmol treated group compared with the 0 nmol treated group.

Patent Claims

1. A therapeutic agent for diseases showing hyponutrition symptoms containing as ghrelin or ghrelin analogue as an effective ingredient.

2. A therapeutic agent in accordance with Claim 1, wherein the disease showing hyponutrition symptoms comprises a disease selected from loss of appetite, cachexia, malignant disease or prostration due to infection or inflammatory disease.

3. A therapeutic agent in accordance with Claim 2 comprising a therapeutic agent for weight loss disease or anorexia accompanied by cachexia.

4. A therapeutic agent in accordance with any of Claims 1-3 comprising peripheral administration.

5. A process for the screening of an agonist or antagonist of ghrelin or an ghrelin analogue including administering a candidate substance to an animal in the presence or absence of ghrelin and the measurement of food consumption, the quantity of NPY mRNA expressed, the bonded quantity of NPY and YI receptor of NPY, oxygen consumption, rate of gastric contents expulsion or activity of vagal nerve.

6. A therapeutic agent for anorexia or the weight loss disease containing an agonist of ghrelin or ghrelin analogue obtained by a process in accordance with Claim 4 as effective ingredient.

7. An preventative agent or therapeutic agent for obesity containing an antagonist of ghrelin or ghrelin analogue obtained by a process in accordance with Claim 4 as an effective ingredient.

FIGURE 1

A

Ghrelin 1: –GSSFLSPEHQRVQQRKESKKPPAKLQPR 28

Motlin 1: FV-PIFTYGELQRMQE-KERNKGQ—— 22

B

```
Ghrelin receptor (GHSR)   1:MWNATPSEEPGFNLTLADLDWDASPGNDSLGDELLQLFPAPLLAGVTATCVALFVVGIAGNLLTMLVVSRFRELRTTTNLYLSSMAFSDLLIFLCMPLDL 100
Motolin receptor          1:M--GSPWNGSDGPEGAREPPWPALP--PC-DERRCSPFPLGALVPVTAVCLCLFVVGVSGNVVTVMLIGRYRDMRTTTNLYLGSMAVSDLLILLGLPFDL 95

Ghrelin receptor (GHSR) 101:VRLWQYRPWNFGDLLCKLFQFVSESCTYATVLTITALSVERYFAICFPLRAKVVVTKGRVKLVIFVIWAVAFCSAGPIFVLVGVEHE---------NGT- 190
Motolin receptor         96:YRLWRSRPWVFGPLLCRLSLYVGEGCTYATLLHMTALSVERYLAICRPLRARVLVTRRRVCALIAVLWAVALLSAGPFLFLVGVEQDPGISVVPGLNGTA 195

Ghrelin receptor (GHSR) 191:----------D-P-W-D-T-NEC--R-P-T-E----FA--VR-S-G-L--LTVMVWVSSIFFFLPVFCLTVLYSLIGRKLWRRRRGDAVVGASLRDQNHKQ 260
Motolin receptor        196:RIASSPLASSPPLWLSRAPPPSPPSGPETAEAAALFSRECRPSPAQLGALRVMLWVTTAYFFLPFLCLSILYGLIGRELWSSRRPLRGPAASGRERGHRQ 295

Ghrelin receptor (GHSR) 261:TVKMLAVVVFAFILCWLPFHVGRYLFSKSFEPGSLEIAQISQYCNLVSFVLFYLSAAINPILYNIMSKKYRVAVFRLLGFEPFSQRKLSTLKDESSRAWT 360
Motolin receptor        296:TVRVLLVVVLAFIICWLPFHVGRIIYINT-EDSRM-MY-FYQYFNIVALQLFYLSASINPILYNLISKKYRAAAFKLLLARKSRPRGFHRSRDTAGEVAG 392

Ghrelin receptor (GHSR) 361:ESSINT----------                                                                              366
Motolin receptor        393:DTGGDTVGYTETSANVKTMG                                                                         412
```

FIGURE 2

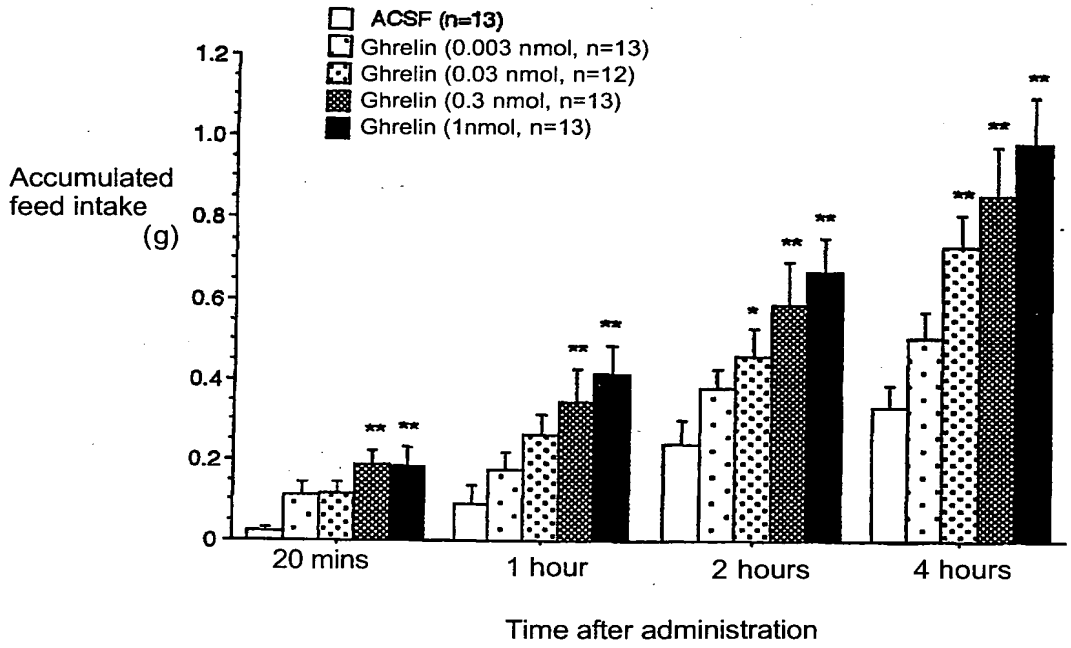


FIGURE 3

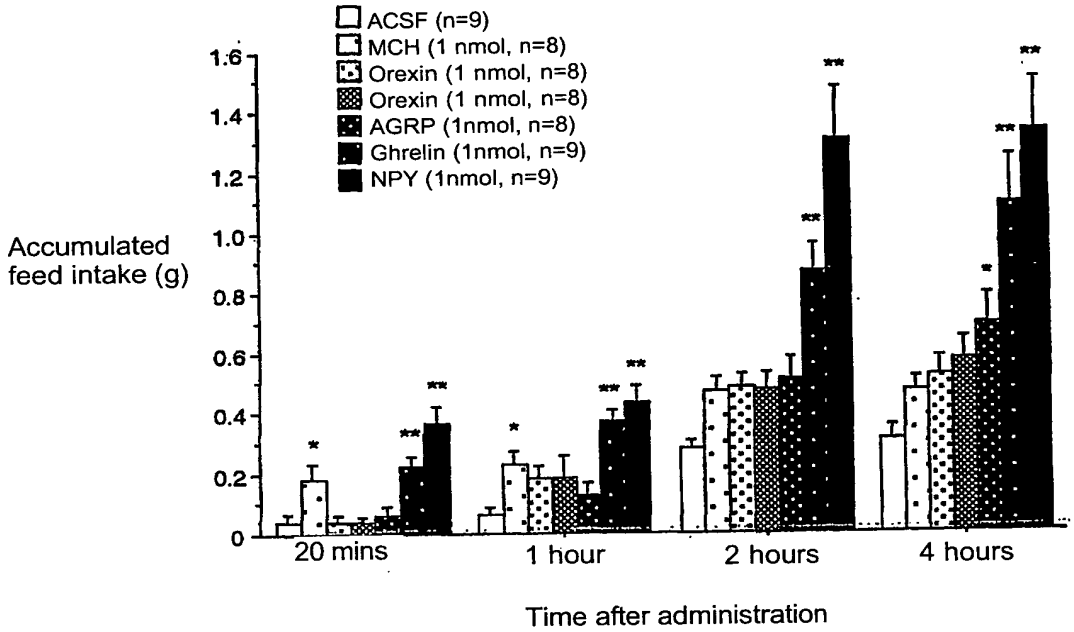

ACSF (n=9)
MCH (1 nmol, n=8)
Orexin (1 nmol, n=8)
Orexin (1 nmol, n=8)
AGRP (1nmol, n=8)
Ghrelin (1nmol, n=9)
NPY (1nmol, n=9)
Accumulated feed intake (g)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
20 mins
1 hour
2 hours
4 hours
Time after administration

FIGURE 4

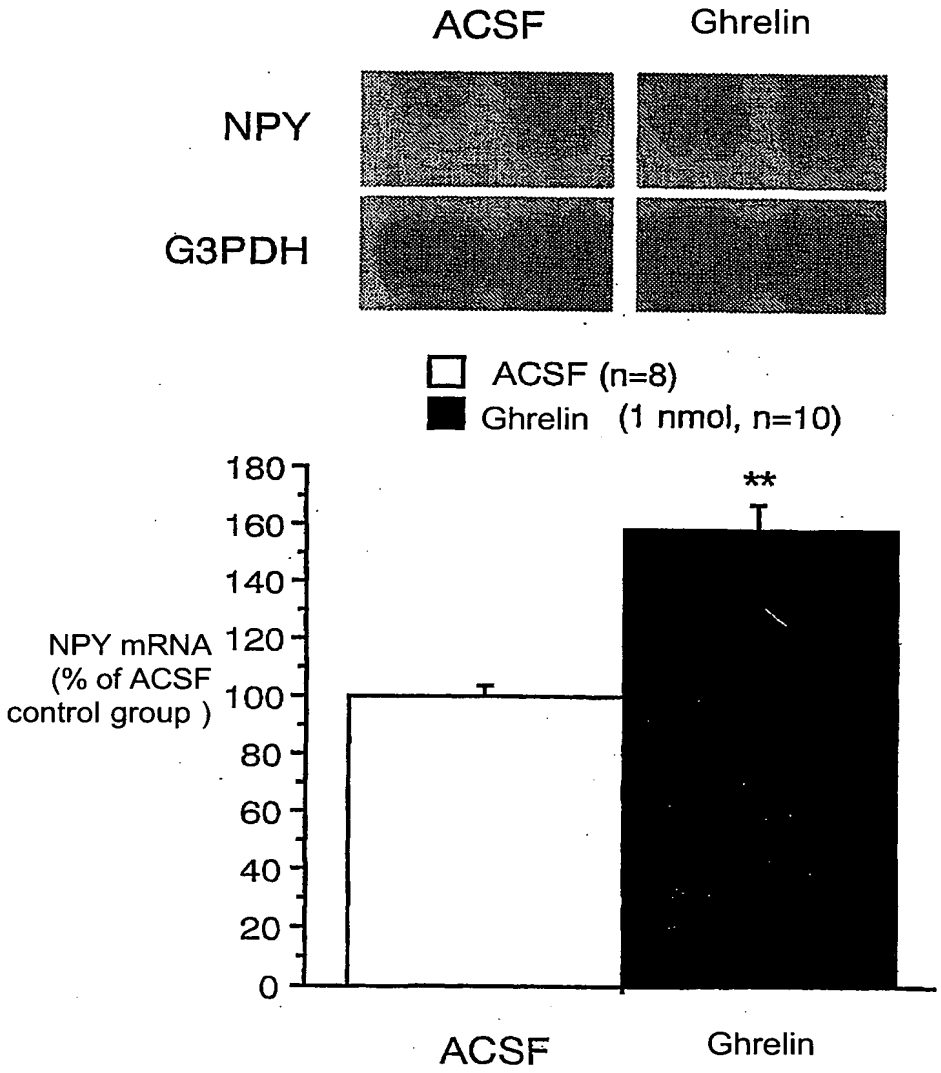


FIGURE 5

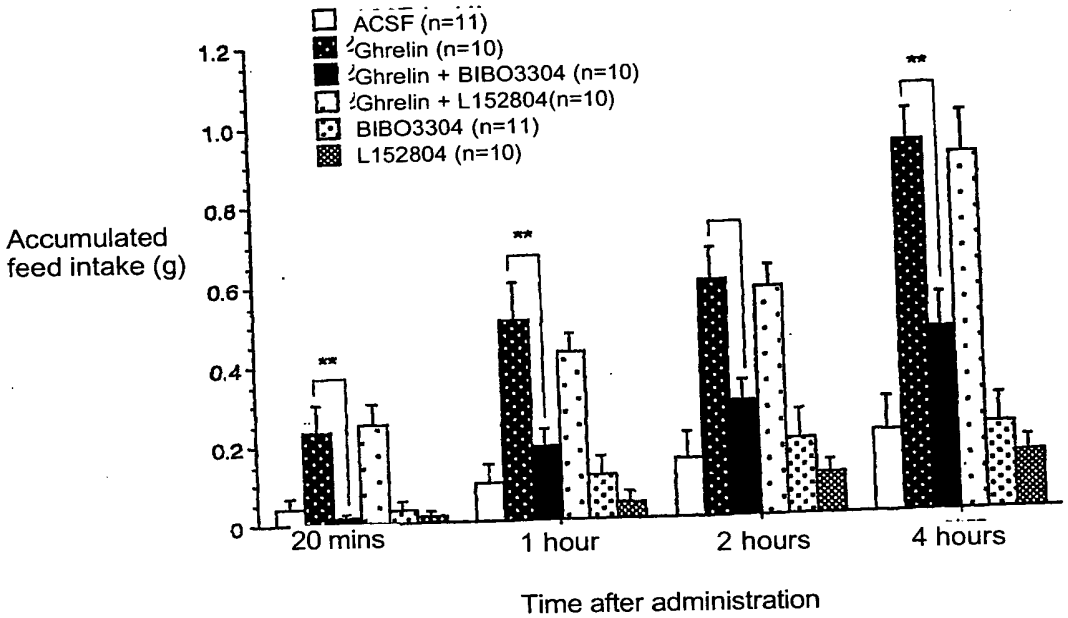


FIGURE 6

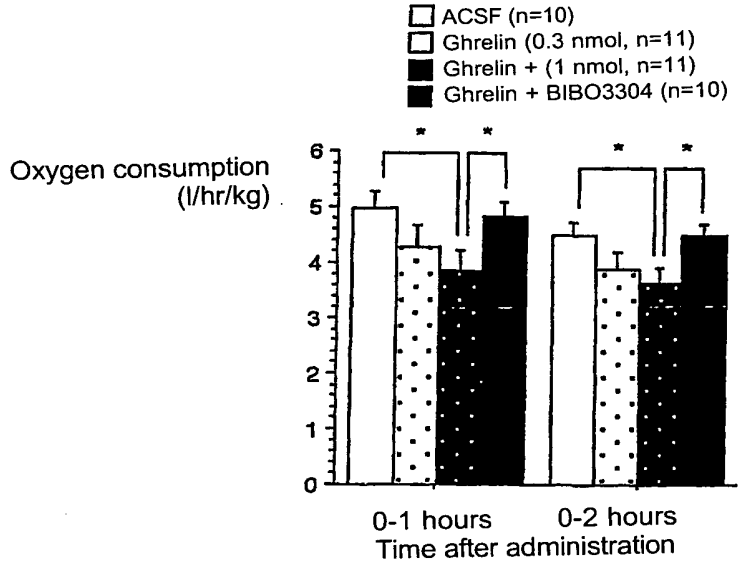


FIGURE 7

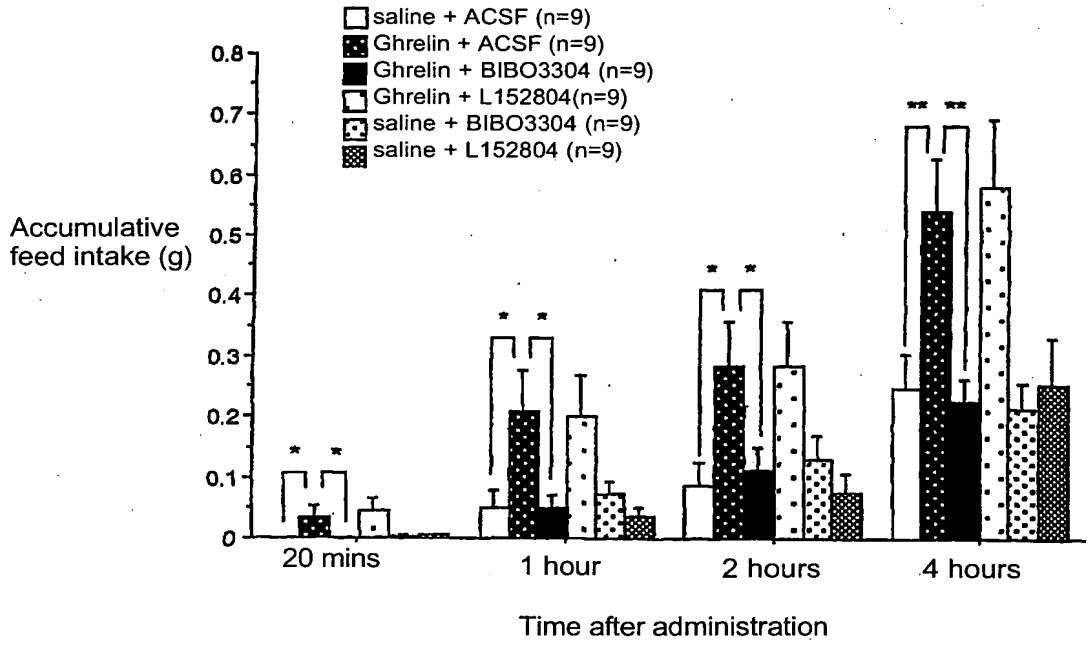


FIGURE 8

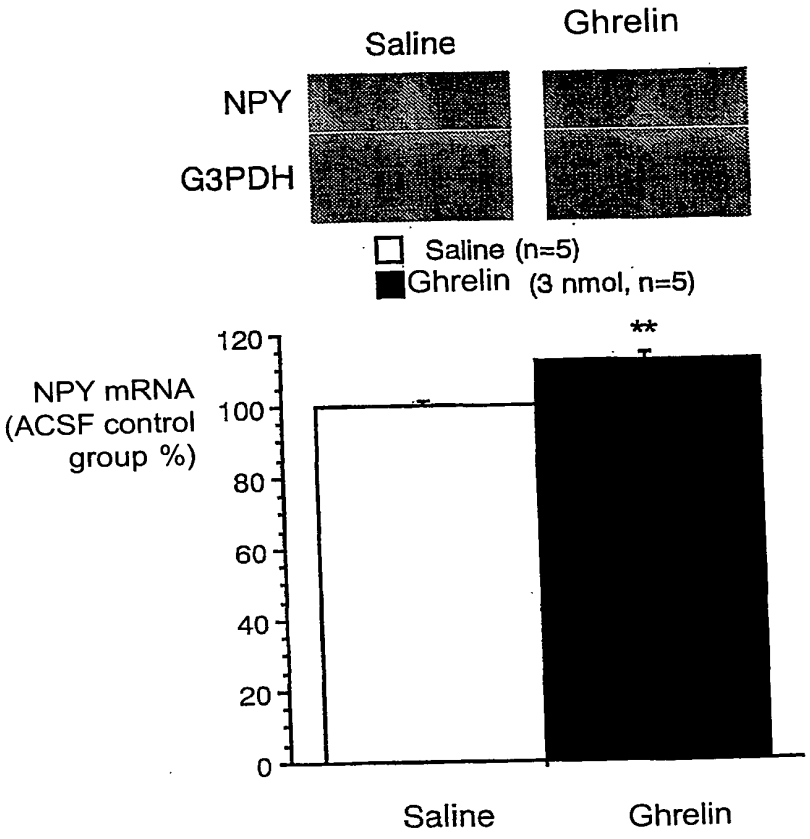

Saline
Ghrelin
NPY
G3PDH
Saline (n=5)
Ghrelin (3 nmol, n=5)
**
NPY mRNA (ACSF control group %)
120
100
80
60
40
20
0
Saline
Ghrelin

FIGURE 9

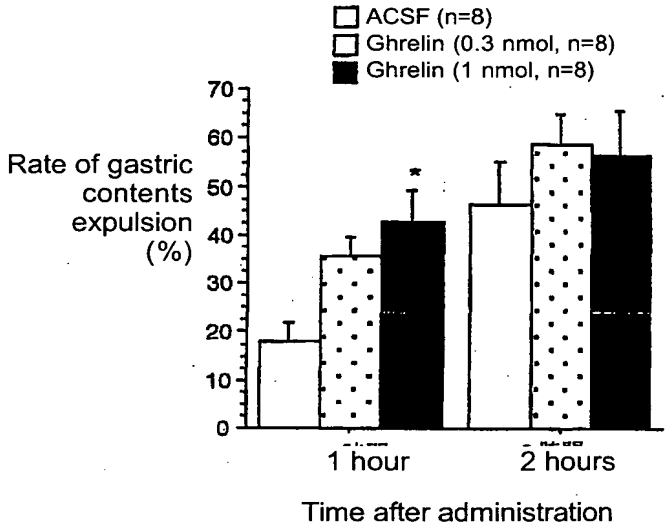


FIGURE 10

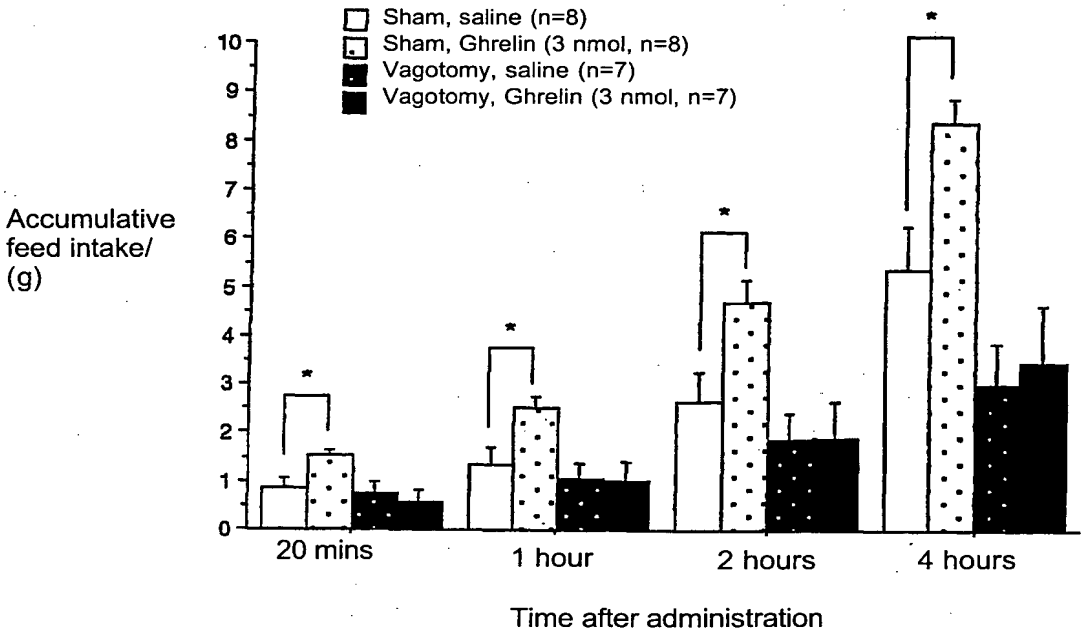
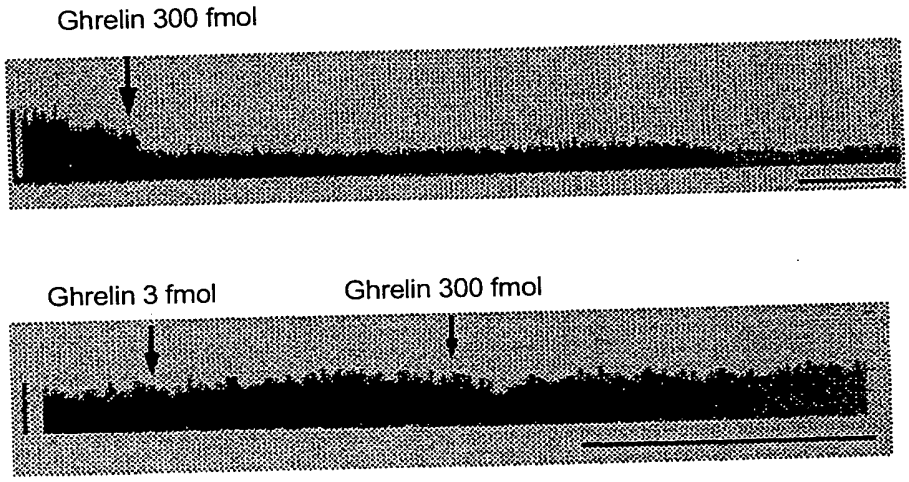


FIGURE 11



Vertical pole : 100 impulses / 5 seconds
Horizontal pole 30 minutes

FIGURE 12

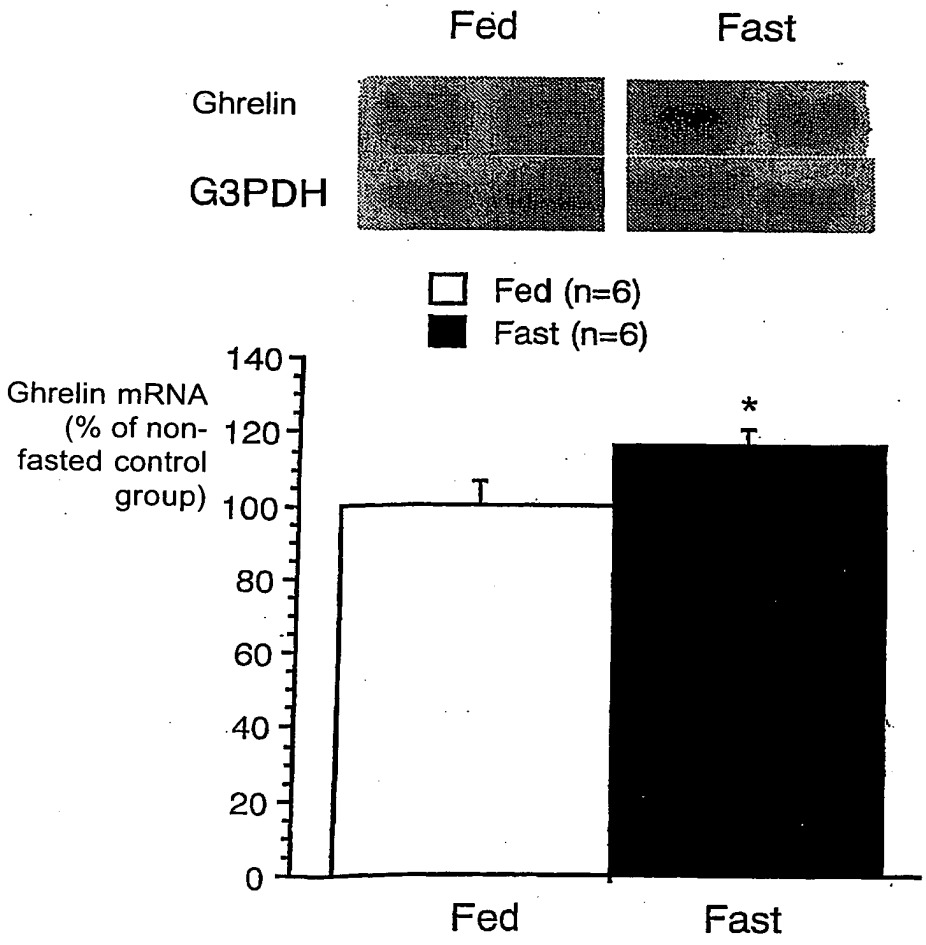

Fed
Fast
Ghrelin
G3PDH
Fed (n=6)
Fast (n=6)
Ghrelin mRNA (% of non-fasted control group)
140
120
100
80
60
40
20
0
*
Fed
Fast

FIGURE 13

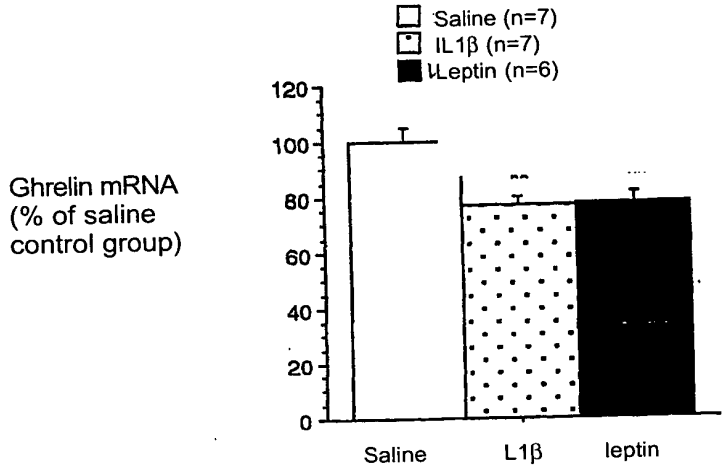


FIGURE 14

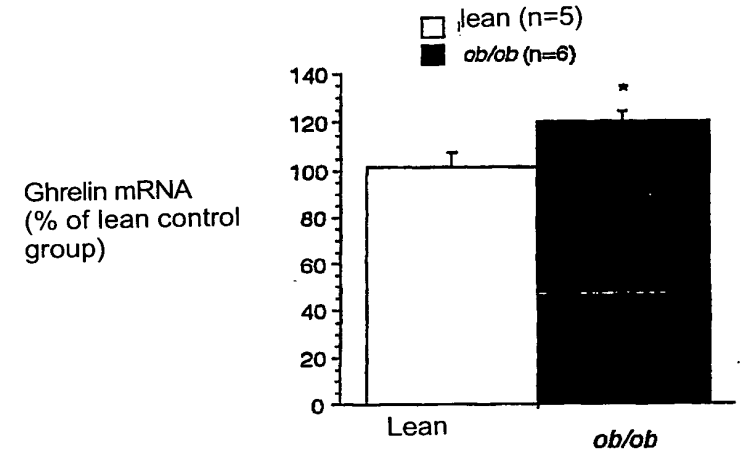


FIGURE 15

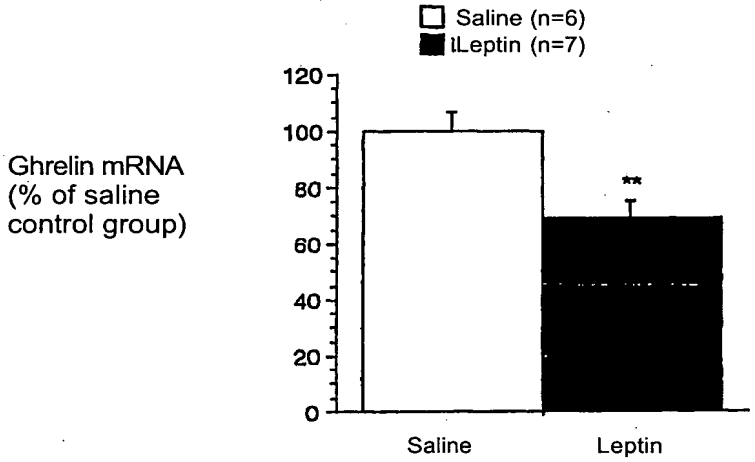


FIGURE 16

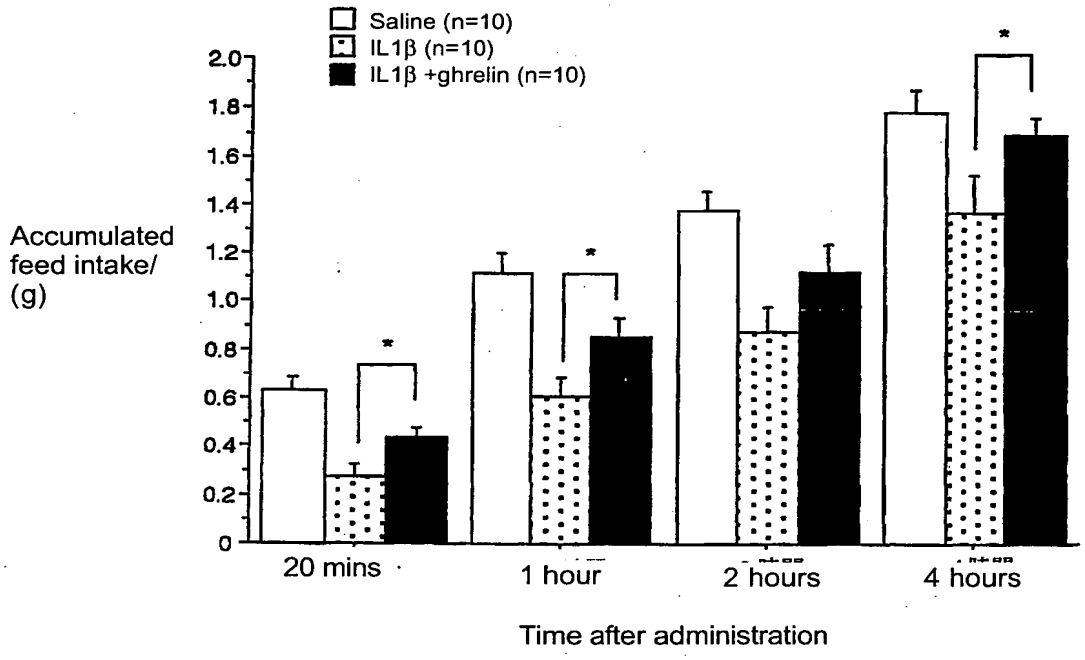


FIGURE 17

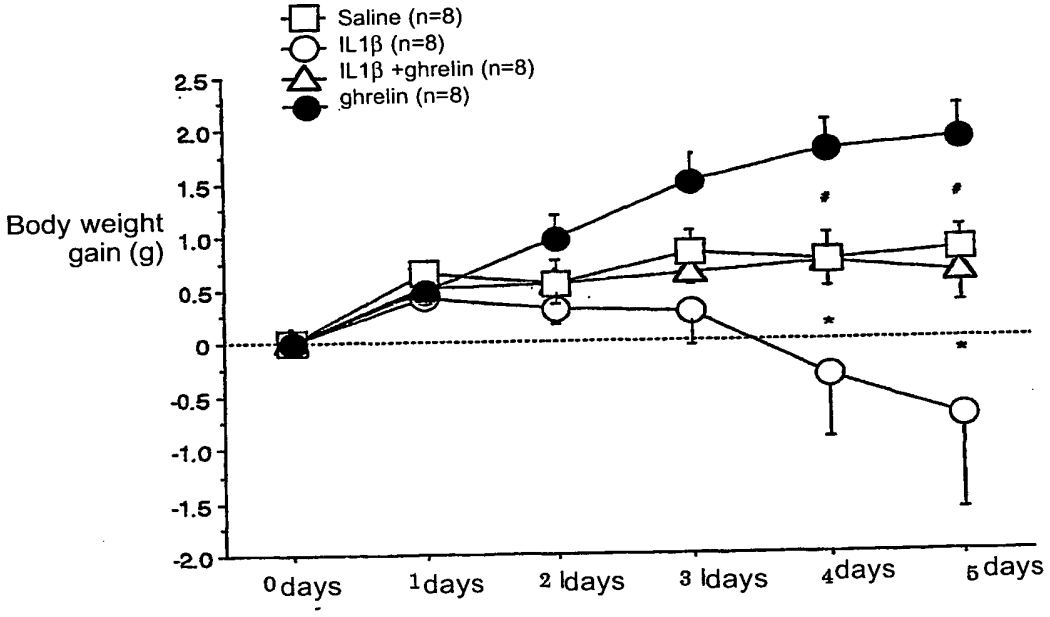


FIGURE 18

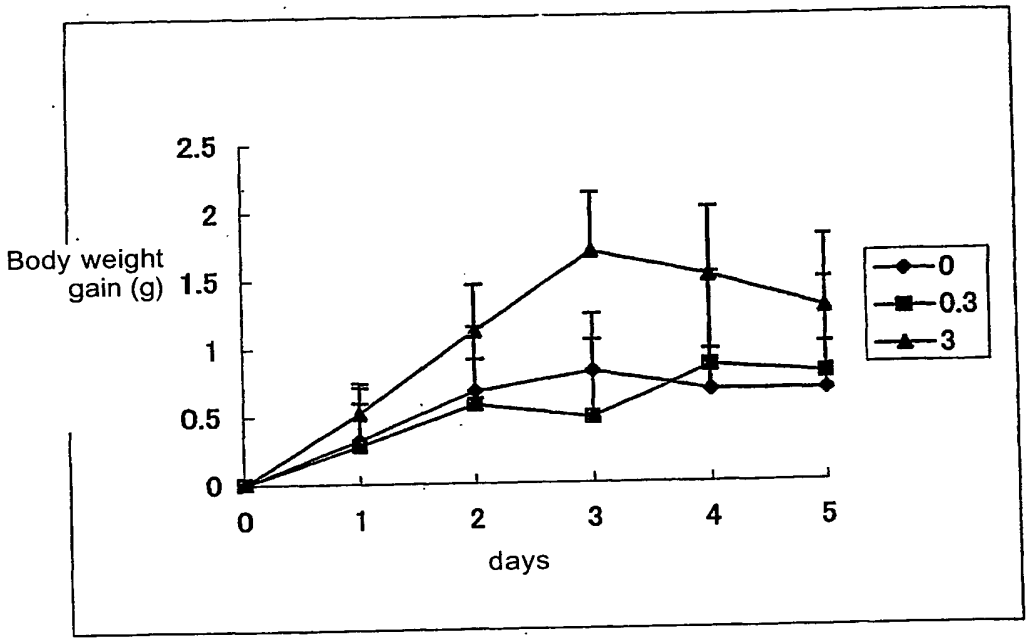
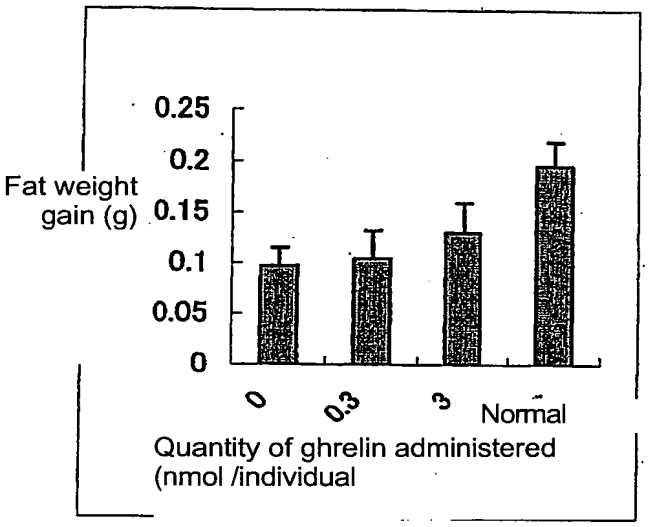


FIGURE 19



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